AD NUMBER

AD866581

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AUTHORITY

BDRL, d/a ltr, 29 Sep 1971

TECHNICAL MANUSCRIPT 594

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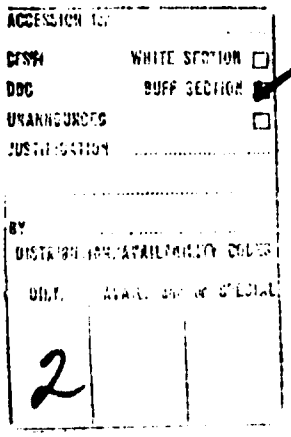
MARCH 1970

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland



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Project 1B061102B71A

March 1970

ACKNOWLEDGMENT

We thank John D. Boyer and Theodore Bryan for skillful technical assistance.

CORRELATION BETWEEN PIGMENT PRODUCTION AND AMINO ACID REQUIREMENTS IN BACILLUS SUBTILIS W-23*

ABSTRACT

Several Bacillus subtilis W-23 auxotrophs cannot produce wild-type pigment normally on minimal agars supplemented sufficiently for growth. This offers a reliable means for scoring genotypes.

This communication presents a convenient tool for distinguishing the prototrophic wild type from several auxotrophic strains of Bacillus subtilis W-23 by differences in colonial pigmentation. Wegman and Crawford[1] described a similar phenomenon for tryptophan auxotrophs of Chromobacterium violaceum. Ulfen[2] characterized his non-pigmented B. subtilis strains as genetically stable, but mentioned no auxotrophic correlations.

Prototrophic colonies of B. subtilis W-23 on minimal 1 agar[3] become red during the second day of 37 C incubation; later they stain the neighboring agar. Several W-23 multiply auxotrophic strains used to study the cotransfer of genetic markers[4] cannot produce pigment normally unless given their amino acid requirements at concentrations above those supporting normal growth. When such strains were transduced so that prototrophs and single auxotrophs appeared together on selective plates, there were mixtures of red and white colonies in the same ratios as prototrophs to single auxotrophs. Replica plating confirmed this apparent correlation, but further study was required to ascertain whether each supplement level that prevented pigment formation by auxotrophs insured recovery of all prototrophic and auxotrophic transformants or transductants. The six double auxotrophs chosen for special study require pairs of seven different amino acids (Table 1, column 1). These strains are those listed by Tyeryar et al.[4] or are other W-23 double auxotrophs similarly developed and characterized. Singly auxotrophic strains, necessary for reconstructed mixtures, were those from which the corresponding double auxotroph had been developed or those derived from that double auxotroph by transduction with phage SP-15 or SP-10.[5] Transduction mixtures[5,6] of strain tyr-2 ile-1 with phage SP-15 provided adequate information for choosing the optimal levels of supplementation indicated in Table 1, but a reconstruction experiment was designed for quantitative work with the other five strains. To insure normal conditions of competition for nutrients with the background population and to produce sufficient numbers of isolated, growing colonies for phenotypic scoring, the reconstruction protocol

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provided that suitable numbers of each combination of genotypes be plated in mixture with the usual concentration of doubly auxotrophic cells on varied levels of each supplement. Care was taken to simulate all aspects of transduction experiments except that the phage was omitted. The selected optimal level of each supplement was always tested in a transduction experiment that included confirmation of the phenotype-genotype correlation by replica plating.

TABLE 1. LEVEL OF NUTRITIONAL SUPPLEMENT THAT PERMITS PHENOTYPIC IDENTIFICATION OF GENOTYPE AND FULL RECOVERY OF ALL PROTOTROPHS AND SINGLE AUXOTROPHS OF BACILLUS SUBTILIS W-23[a]

Genotype of doubly Auxotrophic Strain		Readily Distinguishable from Prototrophs (A^+B^+) by Lack of Pigment after 48 to 72 Hours' Incubation with Optimal Level of Required Supplement		Micrograms of Supplement in each ml of Agar		
					Optimal Level	
A^-	B^-	A^-B^+	A^+B^-	Test Limits	A	B
tyr-2	ile-1	Yes	Yes	12.5 to 200[b]	12.5	50
met-3	ile-1	No	Yes	12.5 to 50	50[c]	50
lys-1	ile-2	Yes	Yes	12.5 to 50	25	50
lys-1	tyr-1	Yes	Yes	12.5 to 50	25	12.5
arg-1	leu-1	Yes	Yes	12.5 to 100	50	50
leu-2	phe-2	Yes	No	12.5 to 100	50	50[c]

a. A^+ = prototrophic and A^- = auxotrophic for nutritional supplement A. Prototrophic colonies, wild-type W-23 in reconstruction experiments and double transductants when phage was used, were usually present on each plate as phenotype controls.

b. Supplements were varied by twofold increments from 12.5 μg to the upper limit tested.

c. The 50 μg/ml requirement for full numerical recovery masked a slight difference in pigment formation. See text.

Table 1 presents the results of these experiments. No level of tyrosine or isoleucine tested affected the frequency of any transductant class (every colony was replicated), but tyrosine concentrations of 25 μg/ml and higher masked the distinction between the tyrosine-requiring, singly auxotrophic transductants and the wild-phenotype, double transductants. Lysine at 25 μg/ml was adequate for full recovery and gave sharper phenotypic differentiation than did 50 μg/ml. The 50 μg/ml level recommended for the other five amino acids was not inhibitory for any genotype. Replication was necessary for reliable genotypic identification on plates supplemented with methionine or phenylalanine (Table 1, footnote c). We concluded that strains tyr-2, ile-1, lys-1, arg-1, and leu-2 can form colonies with less supplement than they require for pigment production on plates covered with large numbers of doubly auxotrophic cells. Figure 1 illustrates the phenotypic contrast on selective plates between colonies whose genotypes were confirmed on replicated plates. Final enumeration and phenotyping are facilitated if colonies are located by an indelible dot on the back of each plate after 20 to 24 hours' incubation, because closely neighboring Bacillus colonies tend to coalesce.

Table 2 permits comparison of the cotransfer data for the tyr-2 and ile-1 markers when genotype identification was by replica plating (Exp. I) and by phenotypical classification (Exp. II) after 48 and 96 hours' incubation. The agreement needs no comment. Although wild-type pigment formation is occasionally delayed beyond 48 hours, error resulting from a 48-hour scoring is probably less than that resulting from genotyping by replication when transductant colonies crowd each other. The few questionable or unexpected phenotypes are readily identifiable by picking to selective media.

Genotype identification on the basis of pigmentation, where applicable, provides a labor-saving alternative to the usual procedure of replicating to selective media. For any given auxotrophic strain, the optimal levels of supplements and time of incubation giving the best phenotypic differentiation should be determined empirically. Mutants such as we have described may also facilitate the study of pigment synthesis in B. subtilis W-23.

FIGURE 1. Example of Phenotypic Identification of Genotypes Occurring in a Transduced Population of the Doubly Auxotrophic B. subtilis W-23 tyr-2 ile-1. Transduction mixtures of recipient cells with phage SP-15 were plated on minimal 1 agar plus 50 µg/ml (A) and 12.5 µg/ml (B). A was replicated (velvet template) to isoleucine-supplemented (A1) and unsupplemented (A2) minimal 1 agar. From B, 16 red and 16 white colonies were transferred with a sterile toothpick to tyrosine-supplemented (B1) and unsupplemented (B2) minimal 1 agar. All the dark (red-pigmented) colonies grew on unsupplemented plates A2 and B2, indicative of prototrophy. The unpigmented colonies on A and B grew on A1 and B1 respectively, but not on A2 and B2, thus demonstrating that they were "single" transductants requiring isoleucine or tyrosine. Plates were photographed after 48 hours at 37 C.

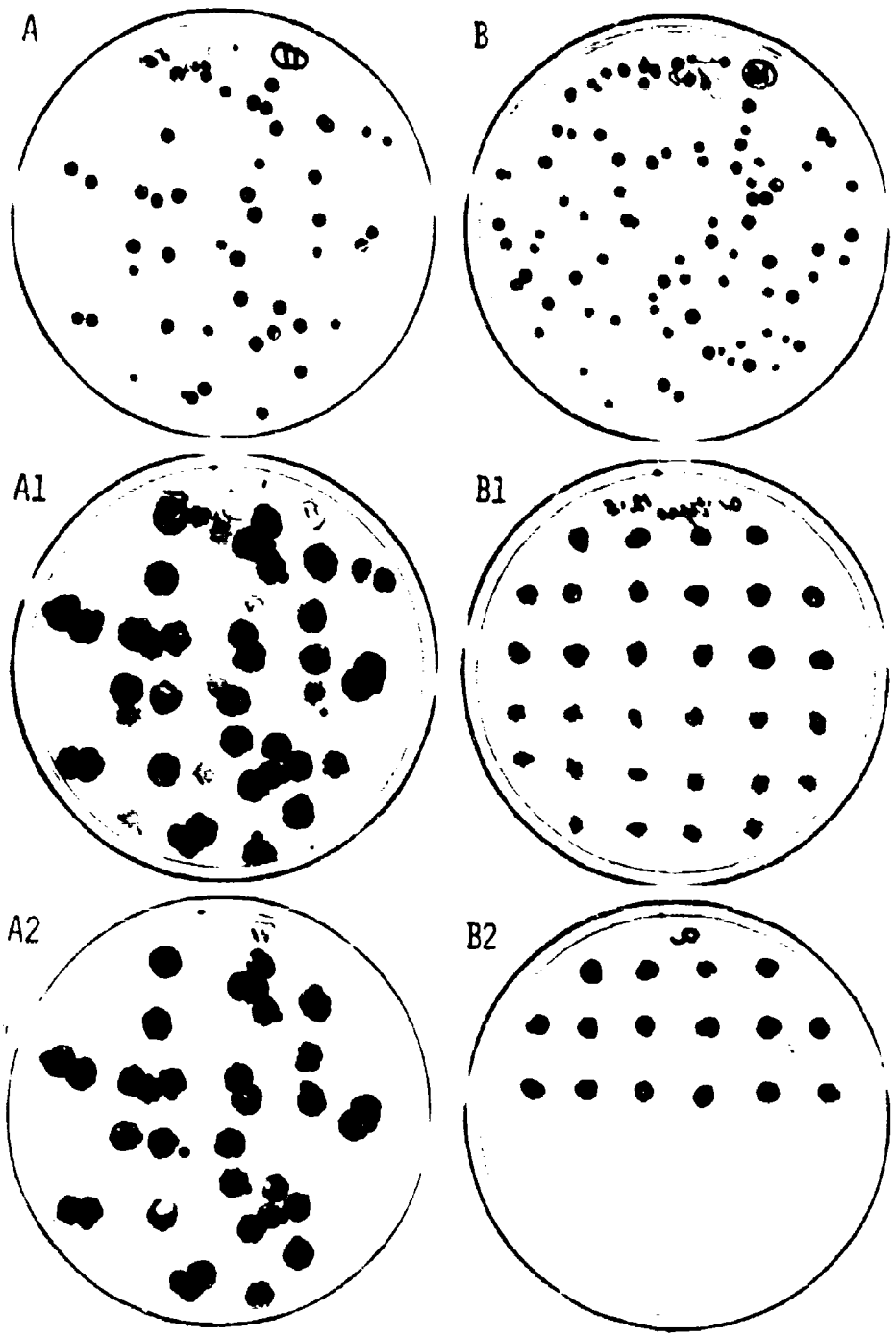
A
B
A1
B1
A2
B2

TABLE 2. COMPARISON OF COTRANSDUCTION PER CENT BASED ON IDENTIFICATION OF TRANSDUCTANT GENOTYPES BY REPLICA PLATING (EXPERIMENT I) AND BY COLONIAL PIGMENTATION (EXPERIMENT II)[a]

Experiment	Transductants			Cotransduction	
	Selected Marker	Number of Colonies	Number of *tyr*$^+$ *ile*$^+$[b]	Per Cent	Average Per Cent
I	*tyr*$^+$	592	426	72.0	58.4
	ile$^+$	923	414	44.9	
II (48 hr)	*tyr*$^+$	676	495	73.2	59.3
	ile$^+$	1483	668	45.0	
II (96 hr)	*tyr*$^+$	676	498	73.7	59.4
	ile$^+$	1483	670	45.2	

a. Phage SP-15 was used to transduce *B. subtilis* W-23 *tyr-2 ile-1*. This double auxotroph was derived independently of the triply auxotrophic recipient, *lys-1 tyr-1 ile-2*, used by Tyeryar et al.[4] to determine cotransduction per cent for *tyr*$^+$ *ile*$^+$. The formula for the calculation was the same as theirs.

b. For Experiment I, all colonies on each selective plate were replicated to confirm that all the red ones were double transductants. For Experiment II, the red colonies after 48 and 96 hours' incubation at 37 C were classified as double transductants.

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Unclassified
Security Classification

DOCUMENT CONTROL DATA - R & D
(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)
Department of the Army
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3. REPORT TITLE
CORRELATION BETWEEN PIGMENT PRODUCTION AND AMINO ACID REQUIREMENTS IN BACILLUS SUBTILIS W-23

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)
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William D. Lawton

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
March 1970	13	6

8a. CONTRACT OR GRANT NO	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO 1B061102B71A	Technical Manuscript 594
c. Task-Work Unit 01-015	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d. DD 1498 Agency Access. DA OL 0167	CMs 6659 SMUFD-AE-T 49805

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
Medical Bacteriology Division	Department of the Army Fort Detrick Frederick, Maryland, 21701

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14. Key Words

Bacillus subtilis
Transduction
Amino acid
Pigment production
Genotype
Identification
Phenotype
Variation
Auxotrophs
Phage SP-15
Phage SP-10

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